View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 581 (2007) 4557–4561

Post-translational modification of barley LTP1b: The lipid adduct lies in the hydrophobic cavity and alters the protein dynamics

Ramani Wijesinha-Bettoni[a], Chunli Gao[b], John A. Jenkins[b], Alan R. Mackie[b], Peter J. Wilde[b], E.N. Clare Mills[b], Lorna J. Smith[a,*]

[a] *Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, UK*
[b] *Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK*

Received 27 July 2007; revised 14 August 2007; accepted 20 August 2007

Available online 29 August 2007

Edited by Sandro Sonnino

Abstract NMR techniques have been used to characterise the effects of a lipid-like post-translational modification on barley lipid transfer protein (LTP1b). NMR chemical shift data indicate that the lipid-like molecule lies in the hydrophobic cavity of LTP1b, with Tyr 79 being displaced to accommodate the ligand in the cavity. The modified protein has a reduced level of backbone amide hydrogen exchange protection, presumably reflecting increased dynamics in the protein. This may result from a loosening of the protein structure and may explain the enhanced surface properties observed for LTP1b.
© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Lipid transfer protein; Post translational modification; NMR; Chemical shift

1. Introduction

Barley non-specific lipid transfer protein 1 (LTP1) is a small 91 residue protein that has been recognised to have a variety of in vitro capabilities including the transfer of lipids between membranes and the inhibition of bacterial and fungal pathogens [1]. Surface properties of LTP1 also play an important role in determining beer foam stability [1]. The protein consists of four α-helices, held together by four disulfide bonds forming a hydrophobic cavity between the helices into which a variety of lipids can bind [1]. The solution structures of both barley LTP1 in an unliganded form and complexed with various ligands have been determined [1].

A post-translationally modified isoform of barley LTP1, named post-translationally modified LTP1 (LTP1b), has been identified in both barley seeds and in beer. There is discrepancy in the literature regarding the nature of the adduct in LTP1b, which is attached to the protein via the side chain of Asp 7. Studies of LTP1b purified from barley grain have proposed *cis*-7-heptadecenoic acid [2]. However, studies which have characterised the protein biosynthesised from linoleic acid have identified the adduct as α-ketol 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid [3]. Here, rather than focusing on the adduct, we concentrate on the protein itself and characterise the ways in which this post-translational modification affects the three-dimensional structure of LTP.

*Corresponding author. Fax: +44 1865 272690.
E-mail address: lorna.smith@chem.ox.ac.uk (L.J. Smith).

Abbreviations: COSY, correlated spectroscopy; DQF, double quantum filtered; LTP1, non-specific lipid transfer protein 1; LTP1b, post-translationally modified LTP1; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy

2. Materials and methods

2.1. Extraction and purification of LTP1 and LTP1b from barley

Barley LTP1b was purified from barley flour (cv. Optic, provided by Brewing Research International, Nutfield, UK) using a combination of ion-exchange followed by gel permeation chromatography based on the method of Lindorff-Larsen et al. [2]. LTP1b containing fractions were pooled and dialysed with 3500 Da cut-off membrane tube against water. LTP1 was prepared using the same method but using barley flour defatted with chloroform/methanol (2:1, v/v).

2.2. Surface activity

The surface pressure was measured using an FTA200 pulsating drop tensiometer (First 10 Å, Portsmouth, VA). A Teflon coated, flat ended tip of 0.94 mm in diameter was attached to a 100 μL syringe and an initial drop volume of 12 μL used. The applied surface area oscillations had a relative amplitude of 5% to avoid excessive perturbation of the interfacial layer, and the measurement frequency was 0.05 Hz. All measurements were made at room temperature.

2.3. NMR studies

All spectra were collected using home-built 600 MHz spectrometers at Oxford University, using 3 mM protein samples in 95/5% H_2O/D_2O. Double quantum filtered (DQF)-COSY, total correlation spectroscopy (TOCSY, 74 ms mixing time), and nuclear Overhauser enhancement spectroscopy (NOESY, 200 ms mixing time) spectra were recorded for LTP1 at pH 4, 37 °C and pH 5, 25 °C. For LTP1b, phase-sensitive correlated spectroscopy (COSY), TOCSY (74 ms mixing time), and NOESY (50 ms, 100 ms and 200 ms mixing times) spectra were recorded at pH 4, 37 °C, and TOCSY and NOESY (74 ms and 200 ms mixing times, respectively) at pH 3, 26 °C and pH 5, 25 °C. For NMR hydrogen exchange experiments the protein was dissolved to 3 mM in 25 mM deuterated sodium acetate buffer (pH 3.6). A series of 6 COSY spectra was collected for each protein at 26 °C. Acquisition was started 1.5 h after dissolution in D_2O and the acquisition time was 7.7 h per spectrum.

3. Results and discussion

3.1. NMR studies

The LTP1 assignments were taken from the published data [4] and were confirmed for the present conditions. The assignments for LTP1b (BioMagRes bank code 15143) were

0014-5793/$32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.08.041

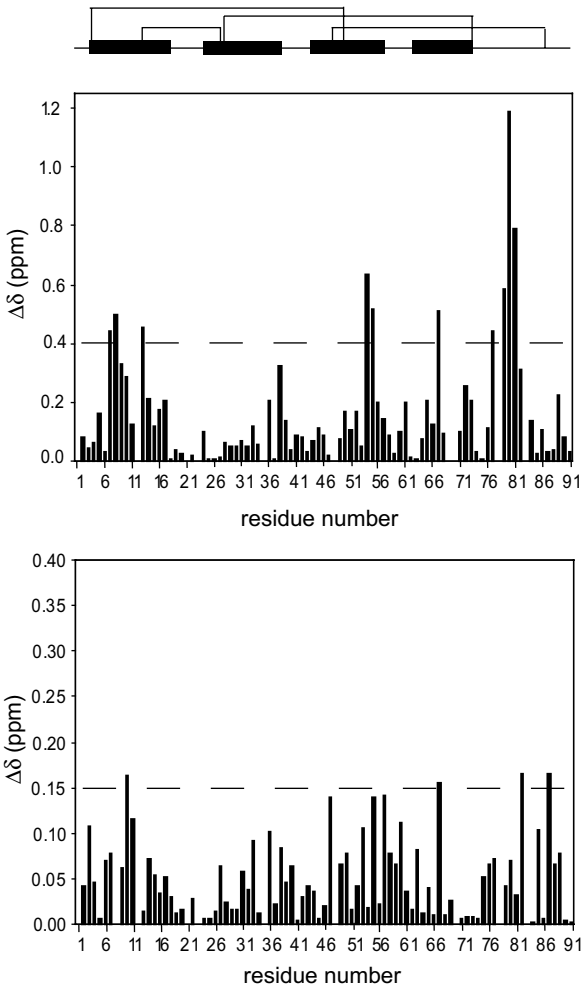

Fig. 1. The absolute values of the chemical shift differences (in ppm) between LTP1 and LTP1b are plotted against residue number for NH (upper panel), and Hα (lower panel). In the case of glycines, only the Hα pair showing the larger difference in chemical shift is shown. α-helical regions and disulphide bridges are indicated above the plot. No data is available for the C48, H35, and Hα of S8 which have not been assigned in LTP1b.

obtained using 2D spectra. Data for LTP1b were of poorer quality than those for LTP1 (see Fig. in Supplementary material). The ^{1}H resonances of the lipid adduct seen in the spectra of LTP1b are in agreement with data quoted by Lindorff-Larsen et al. [2] although the TOCSY connectivity between the end methyl group and the rest of the carbon chain was not observed. From our data it is not possible to distinguish between the two different chemical structures of the adduct proposed previously [2,3]. As most of the lipid signals are in the crowded aliphatic region of the spectrum, it was also not possible to identify any intermolecular NOEs between the lipid and the protein. However, a comparison of the NH and Hα shifts between LTP1 and LTP1b has been performed (Fig. 1). The residues with NH shift differences between LTP1 and LTP1b greater than 0.4 ppm are Asp 7, Ser 8 and Cys 13 (helix 1); Ile 54 and Ala 55 (helix 3); Ala 67 (helix 4) and Val 77, Tyr 79, Thr 80 and Ile 81 (C-terminal region). The differences in Hα chemical shifts are smaller but differences greater than 0.15 ppm are seen for Met 10, Ala 67, Ser 82 and Cys 87. Thus, chemical shift differences are not restricted to the region around Asp 7, where the adduct is attached. Indeed, the most significant chemical shift differences, especially for HN, are seen in the C-terminal region of the protein. Structural analysis shows that most of the residues with significant chemical shift changes cluster around the interior surface of the hydrophobic cavity in the protein (Fig. 2A). This suggests that although the post-translational modification is attached to the side chain of Asp 7 on the protein surface, the lipid-like molecule resides in the internal hydrophobic cavity.

This conclusion is supported by the similarity of the chemical shift changes for LTP1b reported here and those seen for complexes of barley LTP1 with a ligand bound in the hydrophobic cavity, both palmitate and palmitoyl coenzymeA [5,6]. In these studies the main chemical shift changes between free and ligand bound LTP1 were observed for residues in the C-terminal region, residues 77–81 and for residues in helix 3 and the second half of helix 1. Furthermore, the aromatic side chain of Tyr 79 obstructs the hydrophobic cavity in LTP1, and a large difference (0.29 ppm) is seen between the chemical shift of the CβH protons in LTP1 and LTP1b. This suggests that the side chain of this residue may be repositioned in LTP1b to accommodate the adduct within the hydrophobic cavity.

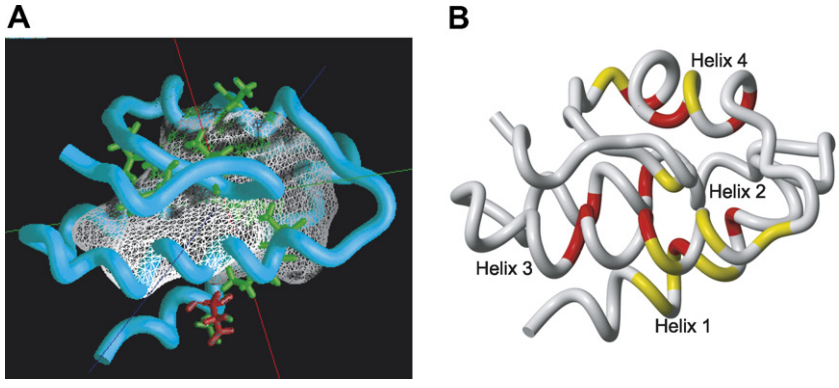

Fig. 2. Structure of barley LTP1 (using PDB entry 1LiP) showing: (A) the residues with chemical shift difference in NH between LTP1 and LTP1b >0.4 ppm (green), Asp 7 where the modification is attached (red) and the hydrophobic cavity in the protein (white mesh); (B) the residues whose backbone amide protons have lower hydrogen exchange protection in LTP1b than in LTP1, yellow: residues protected in both proteins but less protected in LTP1b; red: residues strongly protected in LTP1 and not protected in LTP1b.

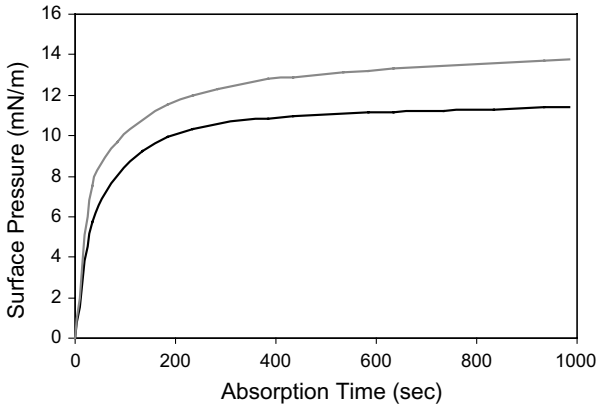

Fig. 3. Surface pressure plotted as a function of adsorption time for LTP1 (black) and LTP1b (grey).

A similar change in the chemical shifts of the CβH protons of the corresponding residue (Tyr81) was also observed in maize LTP1 when complexed with mono acyl phospholipid lysoC16 [7].

The chemical shift data reported here, demonstrating that the adduct lies in the hydrophobic cavity of the protein, are in agreement with previous suggestions by Lindorff-Larsen et al. [2]. However, they are not consistent with a recent model that suggests that a large portion of the ligand in LTP1b is exposed on the surface of the protein [3]. This model is based on a structure of barley LTP1 complexed with three molecules of L-α-lysophosphatidylcholine lauroyl (PDB code 1MID). In this structure two of the ligands lie in the main hydrophobic cavity and a third ligand lies in a third cavity near the C-terminal end of helix 1. It is suggested that the 9 carbon methyl-terminal part of the alkyl chain of the adduct in LTP1b is positioned in this third cavity with the rest of the ligand chain

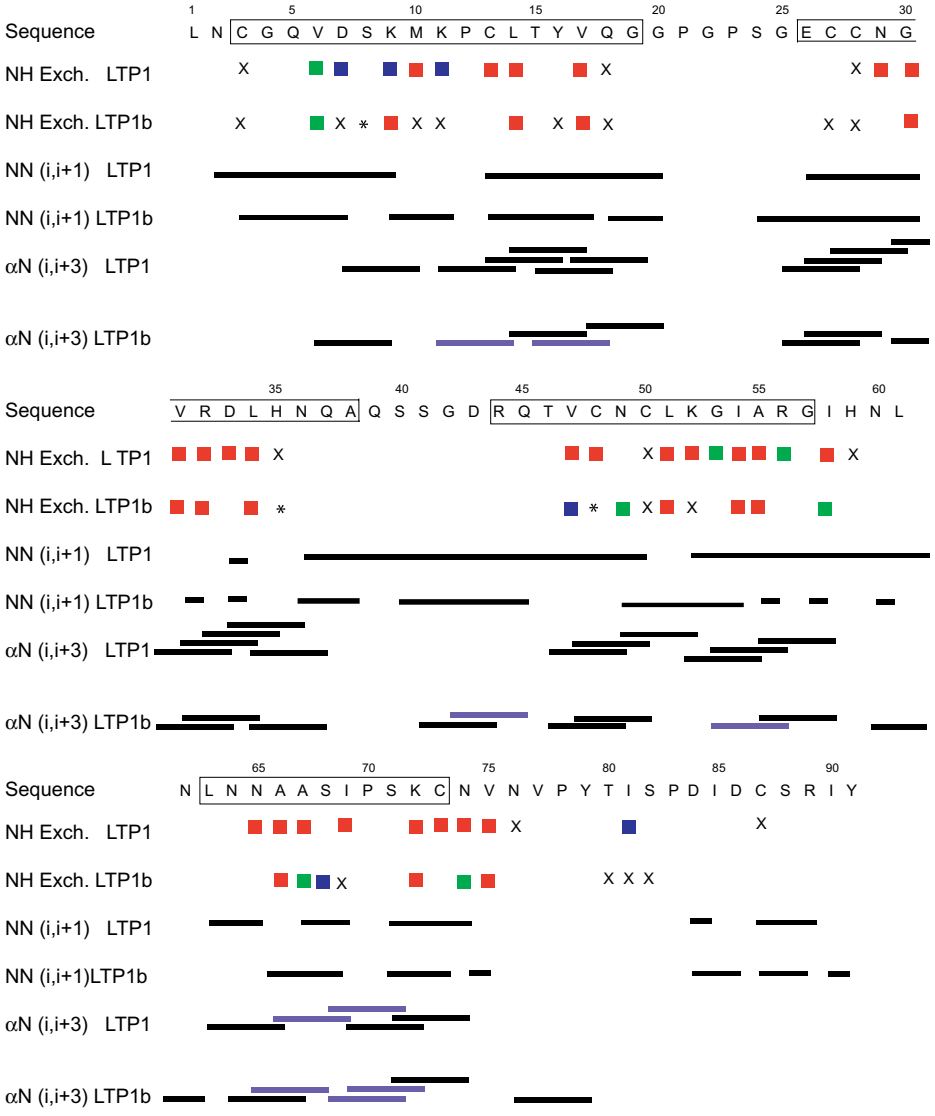

Fig. 4. Hydrogen exchange protection of backbone amide protons and NH–NH($i,i+1$) and αH–NH ($i,i+3$) NOEs identified for LTP1 and LTP1b. Amide proton exchange is shown with blue, green and red squares representing protons present in a spectrum collected 1.5 h, 16.84 h (protected amides) and 39.85 h (strongly protected amides) respectively, after dissolution of the protein in D_2O. Residues marked with an X do not give cross peaks or give very weak cross peaks in a COSY spectrum in H_2O, whilst an * represents unassigned residues. The NOEs are displayed with black lines, blue lines indicating NOEs that cannot be unambiguously identified. Regions of α-helix are framed at the sequence positions.

exposed on the protein surface [3]. This model is used to explain the observation that a monoclonal antibody recognizes LTP1b but not LTP1 and is supported by the observation of narrow linewidths for the adduct resonances in a ^{13}C spectrum of LTP1b.

The data reported here are not compatible with this model for LTP1b, as some of the residues that show significant chemical shift differences between LTP1 and LTP1b would be very distant from the ligand. For example, we estimate that Tyr 79 NH would be approximately 13 Å away from the end of the ligand chain in such a structure. In order to confirm that the chemical shift changes observed for residues in the hydrophobic cavity in LTP1b arise from the lipid-like adduct, and not some other ligand that was retained in the cavity during the normal protein purification procedure, we have further purified a sample of LTP1b. An NMR spectrum of this sample following reverse phase high pressure liquid chromatography and gel filtration was identical to the original spectra.

Further support for a model in which the lipid-like adduct lies in the hydrophobic cavity of the protein comes from analysing the surface properties of LTP1 and LTP1b. A lipophilic adduct would be expected to markedly perturb the surface properties of the protein if located mainly on the surface of the protein. However, comparison of the ability of LTP1b and LTP1 to alter surface pressure at an air:water interface (Fig. 3) shows there is only a modest effect. LTP1b gives a surface pressure of 13.6 mN/m after 15 min compared with 11.4 mN/m for LTP1.

3.2. Hydrogen exchange study

It is interesting to consider if the lipid-like adduct has any effects on the dynamics of the protein structure. To probe this we have studied the hydrogen exchange protection of individual backbone amide protons in LTP1 and LTP1b. The hydrogen exchange protection in LTP1b was on average lower than in LTP1, with significant differences in certain parts of the protein (Figs. 2B and 4). Thus, LTP1 had 32 protected amides, compared with only 20 for LTP1b, the main differences lying in helices 3 and 4. Helix 3 of LTP1 has eight residues with protected backbone amide protons, six of which are strongly protected, whilst LTP1b has only five protected residues, three of which are strongly protected. Similarly, helix 4 of LTP1 has six residues with strongly protected backbone amide protons, whereas LTP1b has four residues with protected backbone amide protons, only two of which are strongly protected. The differences in hydrogen exchange protection are not limited to the area local to the modification (Fig. 2B). We note here that the differences in hydrogen exchange protection in LTP1 and LTP1b are not related to differences in thermal stability since post-translational modification does not alter the resistance of LTP1 to denaturation at temperatures up to 100 °C [8].

Differences in hydrogen exchange protection could reflect structural changes to the α-helical regions in the protein. Inspection of the NOE data for LTP1 and LTP1b showed that for both proteins NH–NH (i,i + 1) and αH–NH (i,i + 3) NOEs are seen, indicative of helical secondary structure, in all the four main regions of α-helix (Fig. 4). Whilst data for LTP1b were of poorer quality, they indicate the secondary structure of LTP1b is not significantly disrupted compared to LTP1. The secondary structure of LTP1 and LTP1b were also almost identical, as determined by Circular Dichroism spectroscopy. However, a number of NOEs are clearly missing from the middle of helix 3 in LTP1b which shows significantly different levels of hydrogen exchange protection in LTP1 and LTP1b. This helix may therefore be more flexible in LTP1b.

4. Conclusions

The post-translational modification of barley LTP1 studied here does not lead to significant changes in either the secondary or tertiary structure of the protein. The chemical shift data for LTP1b suggest that the lipid-like adduct lies in the hydrophobic cavity of the protein. Interestingly its presence appears to alter the dynamics of the protein and gives a loss of hydrogen exchange protection in LTP1b. This change may reflect an expansion of the cavity in the protein core in LTP1b giving a looser packed structure with less rigid helices. The plasticity of the hydrophobic cavity in lipid transfer proteins has been recognised previously, with a significant expansion of the cavity being reported in some complexes. For example, the cavity in wheat lipid transfer protein is reported to increase from 250 $Å^3$ to 750 $Å^3$ on binding di-myristoyl-phosphatidyl-glycerol [9].

The increased flexibility of the protein in LTP1b may be important in allowing multiple molecules to bind within the main hydrophobic cavity. It may also enable the lipid adduct to be relatively mobile within the cavity and hence give rise to the reported narrow ^{13}C NMR resonances [3]. The differences resulting from covalent linkage at the Asp 7 side chain itself, or the changed dynamics of the protein, are likely to be sufficient to allow a monoclonal antibody to discriminate between LTP1b and LTP1 [3] as has been indicated for other antibodies whose binding is determined by protein conformation [10]. The increased dynamics of LTP1b would explain the observed enhanced surface activity of LTP1b compared with LTP1, which could be a direct result of the structural flexibility or as a result of an increase in the mean surface hydrophobicity induced by the increased flexibility as we have found for another model protein, bovine α-lactalbumin [11]. Protein molecules are known to unfold at least partially upon adsorption at an interface [12]. This can cause an increase in the surface hydrophobicity and interactions with neighbouring molecules which in turn decreases the probability that the molecule will desorb back into solution. Therefore, LTP1b molecules may be more effective at stabilising interfaces having a less rigid structure which might be likely to partially unfold more readily on adsorption.

Acknowledgements: We thank Prof. Flemming Poulsen and Dr. Christina Redfield for providing advice on the NMR work. R.W. and C.G. were supported by BBSRC Grants BBS/B/12466 and BBS/B/12393. E.N.C.M., P.J.W., J.J. and A.R.M. acknowledge the support of a competitive strategic grant from BBSRC.

Appendix A. Supplementary data

One figure comparing COSY spectra of LTP1 and LTP1b. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.041.

References

[1] Douliez, J.-P., Michon, T., Elmorjani, K. and Marion, D. (2000) Structure, biological and technological function of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. J. Cereal. Sci. 32, 1–20.

[2] Lindorff-Larsen, K., Lerche, M.H., Poulsen, F.M., Roepstorff, P. and Winther, J.R. (2001) Barley lipid transfer protein, LTP1, contains a new type of lipid-like post-translational modification. J. Biol. Chem. 276, 33547–33553.

[3] Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. and Marion, D. (2006) Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. J. Biol. Chem. 281, 38981–38988.

[4] Heinemann, B., Andersen, K.V., Nielsen, P.R., Bech, L.M. and Poulsen, F.M. (1996) Structure in solution of a four-helix lipid binding protein. Protein Sci. 5, 13–23.

[5] Lerche, M.H. and Poulsen, F.M. (1998) Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins. Protein Sci. 7, 2490–2498.

[6] Lerche, M.H., Kragelund, B.B., Bech, L.M. and Poulsen, F.M. (1997) Barley lipid-transfer protein complexed with palmitoyl CoA: the structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. Structure 5, 291–306.

[7] Gomar, J., Petit, M.C., Sodano, P., Sy, D., Marion, D., Kader, J.C., Vovelle, F. and Ptak, M. (1996) Solution structure and lipid binding of a non-specific lipid transfer protein extracted from maize seeds. Protein Sci. 5, 565–577.

[8] Perrocheau, L., Bakan, B., Boivin, P. and Marion, D. (2006) Stability of barley and malt lipid transfer protein 1 (LTP1) toward heating and reducing agents: relationships with the brewing process. J. Agric. Food Chem. 54, 3108–3113.

[9] Sodano, P., Caille, A., Sy, D., de Person, G., Marion, D. and Ptak, M. (1997) ^{1}H NMR and fluorescence studies of the complexation of DMPG by wheat non-specific lipid transfer protein. Global fold of the complex. FEBS Lett. 416, 130–134.

[10] Tsuiji, H., Xu, L., Schwartz, K. and Gumbiner, B.M. (2007) Cadherin conformations associated with dimerization and adhesion. J. Biol. Chem. 282, 12871–12882.

[11] Wijesinha-Bettoni, R., Gao, C., Jenkins, J.A., Mackie, A.R., Wilde, P.J., Mills, E.N.C. and Smith, L.J. (2007) Heat treatment of bovine α-lactalbumin results in partially-folded, disulfide bond shuffled states with enhanced surface activity. Biochemistry 46, 9774–9784.

[12] Cornec, M., Cho, D. and Narsimhan, G. (1999) Adsorption Dynamics of α-lactalbumin and β-lactoglobulin at air-water interfaces. J. Coll. Int. Sci. 214, 129–142.